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Influence of Bovine Slurry Deposition on the Structure of Nodulating *Rhizobium leguminosarum* bv. viciae Soil Populations in a Natural Habitat

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The population of nodulating *R. leguminosarum* bv. viciae in soil from a grass-covered valley area which had been used for bovine slurry deposition over a period of 5 years was analyzed. For these studies, a rapid and reproducible method based on enterobacterial repetitive intergenic consensus (ERIC)-PCR was applied to identify *Rhizobium* strains which had infected pea nodules. Soil samples were taken from different areas and further analyzed in plant tests to determine the impact of the application of slurry (polluted or nonpolluted), the slope position (summit or toe), and exposure (north or south). After comparison of all PCR fingerprint patterns, 24 strain groups were defined. Some strain groups from the nonpolluted soil were suppressed in the polluted samples, and new strain groups were detected in the slurry-polluted soil. After analyzing relationships between the strain groups, we determined the influences of local factors on the nodulating *R. leguminosarum* bv. viciae population. We show that one of those local parameters, slope position, had significantly greater impact on the composition of the *Rhizobium* population than the presence of slurry.

Various methods have been described to monitor nodulating rhizobial and bradyrhizobial soil populations. During the past 70 years, serotyping has been used as the classical method to investigate presence and distribution of different strain groups (10, 38, 39, 41); however, in recent years, new techniques based on multilocus enzyme electrophoresis (9, 14, 37) and PCR (23, 30, 54) have been developed to characterize strain groups in more detail.

Recently, various families of repetitive (rep) sequences (31), such as the enterobacterial repetitive intergenic consensus (ERIC) sequence, have been used as templates for PCR amplifications (18, 43, 49) to obtain DNA fingerprint patterns for investigation and classification of various *Rhizobium* species (8, 20, 26, 32, 50). A good correlation of strain grouping was observed by different methods of classification, e.g., restriction fragment length polymorphism of amplified variable regions of rDNA sequences, multilocus enzyme electrophoresis techniques, and rep-PCR (11, 26). In addition, strain groups identified by multilocus enzyme electrophoresis could be further distinguished by rep-PCR, as shown for example for *R. leguminosarum* bv. trifolii (26). Hence, the chromosomal localization of rep sequences in *Rhizobium* species indicates phylogenetic relationships between different strains, and represents a relatively simple, but efficient tool for strain classification.

For PCR analysis of nodulating rhizobial strains, it was shown by Pillai et al. (36) that instead of using purified bacterial DNA, crude nodule extracts are suitable for detection of specific genetically marked *R. leguminosarum* bv. phaseolus strains. Furthermore, different research groups have recently shown that nodule extracts can also be used for DNA fingerprinting of infecting rhizobial strains (35, 42).

In this paper, we present a simplified version of the ERIC-PCR technique performed directly on crude nodule extracts. Using this method, we analyze the impact of various environmental parameters on the composition of the population of pea-nodulating *R. leguminosarum* bv. viciae in soil taken from different locations of a slurry-polluted grassland area.

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MATERIALS AND METHODS

Grassland area. To investigate the structure of the *R. leguminosarum* bv. viciae population, we selected a grassland area which had been used for slurry deposition (50 to 100 m^3/ha/year, from May to August) over a period of 5 years (1985 to 1990). Slurry, a bovine excrement mixture with a total amount of solid matter of 5 to 10%, had formed gulleys caused by frequent depositions at the same locations at the slope summit. As a consequence, polluted and nonpolluted areas with different plant communities were directly adjacent to one another. In no case were host plants for *R. leguminosarum* bv. viciae found in the polluted area, whereas at all other sites, horsts of legumes (one horst [5 to 10 plants]/5 m^2) such as *Viciae hirsuta*, *Viciae angustifolia*, *Viciae tetrasperma*, and *Medicago lupulina* were dispersed.

Sites and soil samples. In May 1993, soil samples (loamy sand) were taken from slurry-polluted and nonpolluted areas from different slope positions (summit or toe) as well as different exposures (north or south) for examination. From each of the eight variants (slurry polluted, slope summit, north; slurry polluted, slope summit, south; slurry polluted, toe of slope, north; slurry polluted, toe of slope, south; nonpolluted, slope summit, north; nonpolluted, slope summit, south; nonpolluted, toe of slope, north; nonpolluted, toe of slope, south), five independent soil samples (0 to 30 cm deep) from an area of 1 m^2 were taken and pooled for further analysis.

The soils were analyzed previously for potassium, phosphate, magnesium, total carbon, and nitrogen (17). The most important differences between nonpolluted and polluted soils can be summarized as follows (in milligrams per kilogram of soil): potassium, 100 and 300; magnesium, 75 and 120; mineral nitrogen, 20.40 and 37.20. In addition, a significantly increased amount of magnesium (50 mg/kg more) was found in soil samples derived from the toe of the slope. No significant differences were determined for total carbon (15,000 ± 2,000 mg/kg). As determined by electrometric measurements of potassium chloride soil extracts, the only samples showing a pH of 5.0 were those taken from the slurry-polluted northern exposed toe of the slope. For all other samples, pH 7.4 was determined.

Plant tests with soil samples. Three pots (diameter, 15 cm) per soil sample (eight samples in total [see above]) were prepared, containing 500 g of the respective soil and two plant seeds: one seed of *Pisum sativum* cv. Grapis and one seed of cv. Frogel, which were surface sterilized as described by Vincent (51). After 5 weeks of growth under greenhouse conditions, the nodules were harvested, washed three times in sterile water, and frozen separately at −80°C. Six nodules per plant (36 nodules per soil sample) were analyzed by ERIC-PCR.

Plant inoculation test. The following *R. leguminosarum* bv. viciae strains were used: B10 (22), G122 (47), E163N (47), VF39Sm (19), 3894 (3), and pis4, pis5,

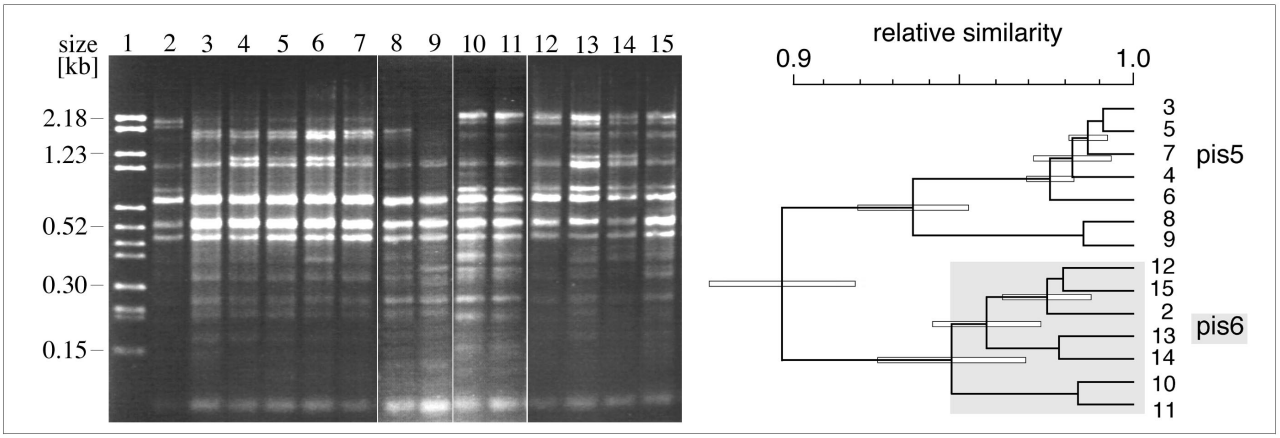

FIG. 1. Variation of ERIC-PCR fingerprint patterns of two similar *R. leguminosarum* bv. viciae strains from four independently performed experiments and the resulting classification. The electrophoretic patterns of different nodule extracts obtained after inoculation with pis6 (lanes 2 and 10 to 15) or with pis5 (lanes 3 to 9) are shown. Lane 1 shows the DNA standard marker (pBR328; *Hin*fI-*Bgl*I). The lane numbers in the gel image correspond to the numbers in the dendrogram. The error bars (double standard deviation) of the calculated similarities are indicated within the tree.

and pis6 (G. Höflich, Zentrum für Agrarlandschafts- und Landnutzungsforschung, Müncheberg, Germany). All strains were grown in TY liquid medium (2) or on solid *Rhizobium* minimal medium (1). The inoculation was performed by adding 10^6 cells of the respective *R. leguminosarum* bv. viciae strain liquid culture per Leonard jar (51). The surface-sterilized seeds of *P. sativum* cv. Grapis were pregerminated in the dark for 3 days (51). The shoots were transferred to the inoculated Leonard jars containing standard nutrient solution (51) and were grown under supplemental light (climate chamber). The plant nodules were harvested and frozen as described above.

PCR. For template preparation, either single colonies grown on solid medium or nodules were used. To prepare total DNA, a single bacterial colony was picked, resuspended in 20 μl of 25 mM NaOH–0.25% sodium dodecyl sulfate, and heated for 15 min at 95°C. Nodules were crushed and treated similarly. For one PCR, 0.2 μl of the lysate was used. Both primers applied in this study are homologous to the ERIC sequence and have been described previously (49). The amplification was performed as described by de Bruijn (8) but with the extension performed at 72°C. The PCR products (3- to 5-μl samples) were separated on 2% Metaphor agarose (FMC) gels under identical conditions. The gels were stained with ethidium bromide and documented by a video camera image system (EasyImage Plus; Herolab). All amplifications were performed twice, at two independent time points. If the patterns were identical, one copy was added to the database for population analysis. *Hin*fI-*Bgl*I double-digested pBR328 DNA (Boehringer Mannheim) was used as size standard and was loaded twice per gel.

Population analysis. By using the software program GelCompar 3.0 (Applied Maths; Herolab), the patterns of single tracks of all gels were analyzed by conversion, normalization, and comparison. The most important steps can be summarized as follows. Step 1 was conversion, which involved loading the gel image (taken by EasyImage Plus) and calculating densitometric track curves. The conditions were a spline thickness of 5 and track resolution of 500. Step 2 was normalization, which involved using both standard marker tracks to even out any smiling effect of the gel and rescaling all gels to one standard marker lane selected as the reference track. The resulting densitometric curves of the tracks were added to the database. The normalization settings were as follows: smoothing, 5; normalized gel resolution, 500; block range, 10% of the complete track. Step 3 was comparison, which involved correction of densitometric curves by elimination of peaks with a relative peak area smaller than 0.5%. Of these corrected densitograms, a range of fragments between 0.3 and 3.0 kb in length were used for automated cluster analysis in accordance with the Ward algorithm (52). The Ward algorithm minimizes the overall deviation of the phylogenetic tree from the original matrix of similarities.

RESULTS AND DISCUSSION

PCR from root nodule extracts. For rapid analysis of nodule-infecting *R. leguminosarum* bv. viciae strains, a method based on ERIC-PCR was simplified by using crude extracts from nodules directly for PCR. To test the reproducibility of this method, seven different strains were used for plant inoculation tests. All strains were identified unambiguously in nodules of the corresponding infected plant, and the resulting fingerprint patterns were identical to those obtained from the original bacterial strain (data not shown). This is in a good agreement with the results of Louws et al. (30), who have shown for *Pseudomonas* and *Xanthomonas* species that the rep-PCR fingerprint profiles are independent of cultivation conditions and DNA purification steps. To obtain evidence for the accuracy of the methods (nodule preparation, ERIC-PCR, pattern analysis by GelCompar) used to identify a specific strain, at least seven DNA patterns obtained independently from different nodules infected with the same strain were compared. A pattern similarity of greater than 94% (±1.5%) was calculated. Two of the seven strains possessed very similar patterns (pis5 and pis6). As illustrated in Fig. 1, these two strains could be distinguished from each other after calculation of the corresponding fingerprint patterns.

To investigate the specificity of the technique for detecting the infecting strain of a single nodule, a 1:1 culture mixture of *R. leguminosarum* bv. viciae B10 and G122 was chosen for inoculation of *P. sativum* cv. Grapis. In all cases, it was demonstrated that the ERIC-PCR pattern of only one strain per nodule was detectable. Since strain B10 is more competitive than strain G122 (data not shown), 92% of the nodules investigated were occupied by B10. We found no hint of a doubly infected nodule.

Tests with experimentally mixed extracts from nodules infected with either B10 or G122 showed that at least 20% of one nodule extract must be added to the other one before a significantly altered PCR fingerprint pattern is obtained. For the population analysis, we concluded that if mixed infections occurred in the field, only nodule inhabitants representing more than 20% of the nodule population would change the band pattern. In such cases, it is quite possible that mixed nodule infections caused by different amounts of the same strains resulted in patterns which were classified as separate strain groups by the GelCompar program.

On the basis of data obtained from the pattern similarity tests (see above), which reproducibly resulted in a 92.5% (94% ± 1.5%) similarity as the upper limit, fingerprint patterns with similarities greater than 92% were defined as belonging unambiguously to the same strain group. A similarly high degree of differentiation of fingerprint patterns by GelCompar has been described in a recent publication (48). In comparable experiments, Haukka and Lindström (16) and van Rossum et al. (48) also analyzed band patterns. The data for each band, which included peak positions and peak areas, were based on densi-

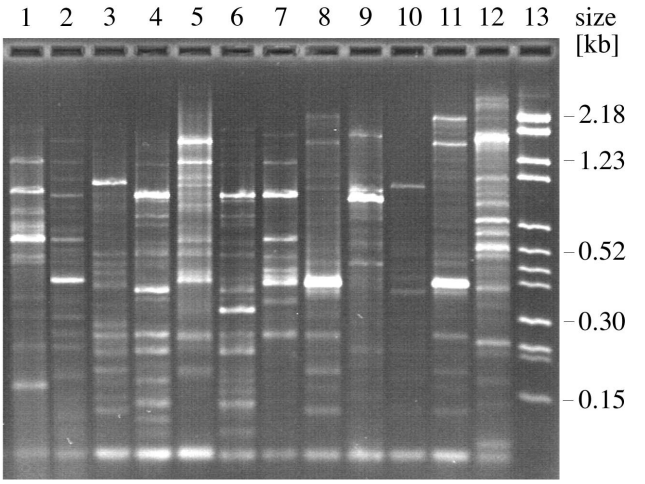

FIG. 2. Example of ERIC-PCR fingerprint patterns of nodules harvested from pea plants grown in three different soil samples originating from the grassland experiment. Lanes: 1 to 4, polluted soil (south-exposed slope summit); 5 to 8, polluted soil (south-exposed toe of slope); 9 to 12, nonpolluted soil (north-exposed toe of slope); 13, standard marker (pBR328; *Hin*fI-*Bgl*I).

tometric curves obtained by scanning the respective gel tracks. On the basis of results obtained by comparison of the very similar strains pis5 and pis6 (Fig. 1), which could not be distinguished by using only peak positions for calculation, it can be concluded that pattern calculations based on densitometric curves are much more sensitive and allow a more precise definition of different strain groups. To date, for rhizobial population studies based on ERIC-PCR, only peak positions have been compared and calculated (20, 32).

***R. leguminosarum* bv. viciae population analysis.** To investigate possible effects of bovine slurry on the nodulating *R. leguminosarum* bv. viciae population structure in soils from different locations in the valley, each soil sample was used for plant tests under greenhouse conditions as described in Materials and Methods. Any effect of moisture and temperature that might differentially influence the rhizobial population (13, 25, 33, 34, 53, 56) was thus avoided. Therefore, detectable differences in population structure should be caused by chemical parameters of the soil. Figure 2 shows an example of the variation of *R. leguminosarum* bv. viciae fingerprint profiles obtained from nodules of plants grown in three different soil samples. After GelCompar analysis of all ERIC-PCR fingerprints (288 in total), 24 strain groups were established based on a similarity level of 92%. This level of diversity of the nodulating *R. leguminosarum* bv. viciae population is comparable to that found in soil from different locations by using other methods of genetic fingerprinting (5, 6, 15, 24, 55). It is quite possible that this is due to the host plant, which might select only a certain number of certain types of strain groups in the soil. The proportion of each of the 24 groups was in no case more than 5% of the total number of patterns analyzed (Table 1). In contrast to earlier rhizobial population structure analysis (27, 28, 40), no dominant strain groups could be detected. Of the 24 strain groups, 19 were derived from nodules isolated from both plant cultivars used for the nodulation tests. Only a few strain groups seemed to be cultivar specific (Table 1). Therefore, we suggest that one cultivar is sufficient to trap a representative part of the natural nodulating population. This assumption is supported by the findings of Strain et al. (45), who have shown that there is no significant relationship between the identified *R. leguminosarum* bv. viciae population occupying nodules and host plant species.

The strain groups were also examined with regard to their site of origin (slope position, slope exposure, slurry treatment). We looked for characteristics typical of all members of one specific group, and if such characteristics were found, the spe-

TABLE 1. Typical characteristics of the resulting *R. leguminosarum* bv. viciae strain groups identified in the grassland area

Strain group	Internal similarity (%)[a]	Relative portion (%)[b]	Typical characteristic	Pea cultivar used
1	91.8 ± 1.2	4	With slurry	Grapis and Frogel
2	92.6 ± 1.6	4	South	Grapis and Frogel
3	91.8 ± 0.9	3	Toe of slope, south	Grapis and Frogel
4	93.1 ± 3.5	3	Summit	Grapis and Frogel
5	91.2 ± 2.1	4	None	Grapis and Frogel
6	94.0 ± 1.1	4	Toe of slope, north, with slurry	Grapis and Frogel
7	93.3 ± 2.1	5	Summit, south	Grapis and Frogel
8	93.2 ± 1.1	5	Without slurry	Grapis and Frogel
9	92.5 ± 1.7	4	Summit	Grapis and Frogel
10	92.6 ± 2.1	5	Summit, without slurry	Grapis
11	92.8 ± 1.7	4	North, without slurry	Grapis
12	92.5 ± 2.5	5	North, with slurry	Grapis and Frogel
13	91.0 ± 1.7	5	None	Grapis and Frogel
14	94.2 ± 1.2	4	With slurry	Grapis and Frogel
15	92.0 ± 2.5	4	With slurry	Grapis
16	92.7 ± 2.2	3	Summit, without slurry	Frogel
17	91.4 ± 1.6	5	Toe of slope, without slurry	Grapis and Frogel
18	92.5 ± 2.5	5	Toe of slope, without slurry	Grapis and Frogel
19	92.1 ± 1.5	3	Toe of slope, north, without slurry	Frogel
20	92.9 ± 2.5	5	Toe of slope	Grapis and Frogel
21	93.2 ± 1.7	3	Toe of slope, north	Grapis and Frogel
22	92.0 ± 1.7	5	Toe of slope, south	Grapis and Frogel
23	92.4 ± 1.2	5	Without slurry	Grapis and Frogel
24	93.7 ± 2.2	3	Toe of slope, north, without slurry	Grapis and Frogel

[a] The internal similarities of the fingerprint patterns of the specific strain group are given with standard deviations.
[b] The relative portions were calculated by comparing the number of fingerprint patterns of the group with respect to all patterns analyzed.

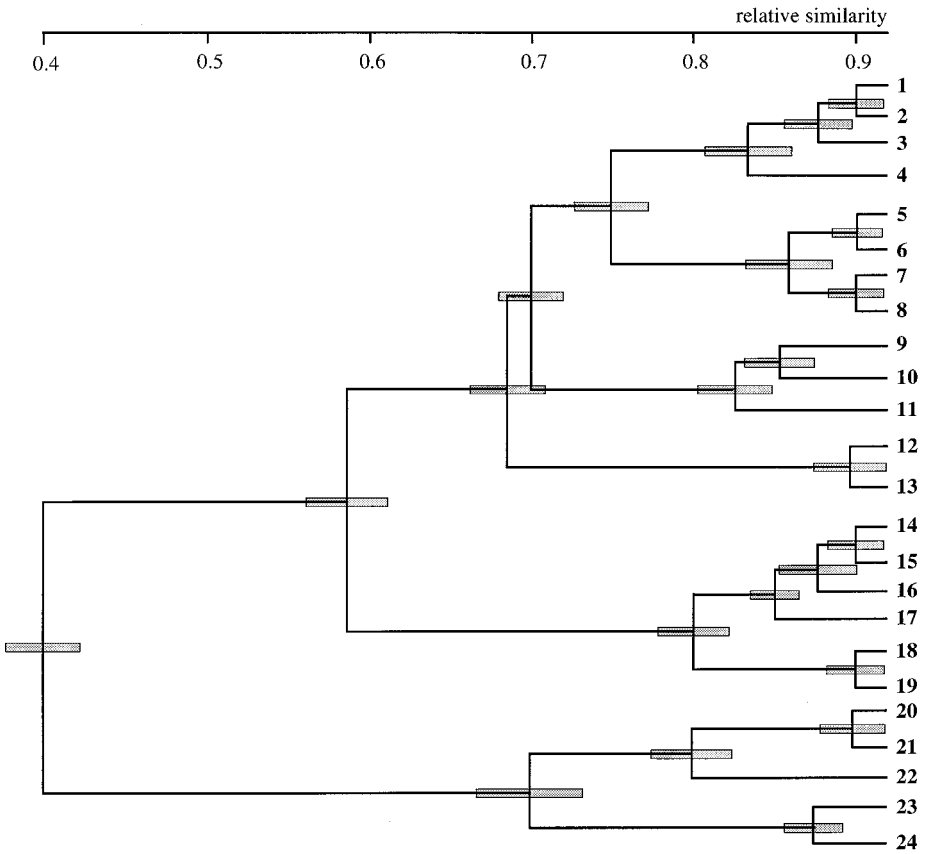

FIG. 3. Phylogenetic tree characterizing the relationships between the strain groups after cluster analysis of ERIC-PCR patterns obtained from all soil samples. The strain group numbers (1 to 24) are identical to those listed in Table 1. The error bars (grey) represent double standard deviations of the calculated branches.

cific group was assigned accordingly (Table 1). Comparison of strain groups found in nonpolluted and slurry-polluted soil samples indicated that 9 of the 19 strain groups present in nonpolluted soil samples were not detected among isolates from slurry-polluted soils (data not shown). Additionally, five strain groups were found only in soil treated with slurry.

The phylogenetic relationships of the 24 strain groups identified in the grassland experiment, including double standard deviations to estimate significant differences, are illustrated in Fig. 3. Each branching point of the tree was screened for identical marks by comparing all 288 fingerprint patterns. We found that 57% of the patterns belonging to strain groups 1 to 19 originated from soil samples taken from the summit, independent of exposure and pollution, and 82% of the patterns belonging to strain groups 20 to 24 originated from soil samples taken from the toe of the slope. Here too, no differences between exposure and pollution were found. The division into these subpopulations occurred at a relative similarity of 0.4 (Fig. 3). By comparing the fingerprint patterns of strain groups 20 to 24, a branching point was identified at a relative similarity of 0.7, which was correlated with slurry pollution. Whereas 60% of the patterns belonging to groups 20 to 22 originated from slurry-polluted soil samples, all patterns of strain groups 23 and 24 were derived from nonpolluted samples (Fig. 3). Additionally, patterns belonging to strain groups 9, 10, and 11 reflect a subpopulation originating from nonpolluted soil samples. The corresponding branching point also was found at a relative similarity of 0.7.

An early division into subpopulations is expected to have been caused by factors with a strong effect on the population structure. Since it has previously been shown for *R. leguminosarum* bv. viciae that the presence of a host plant has no influence on the diversity of the rhizobial population in the field (45), we assume that in our experiments the few host plants growing in the grassland area (e.g. *Viciae hirsuta*; see Materials and Methods) did not exert a major effect on the genetic diversity. Therefore, the earliest branching (relative similarity, 0.4) probably is caused mainly by soil parameters affected by different slope positions. Attempts to correlate various parameters (pH, carbon, nitrogen, potassium, phosphate, and magnesium) led to the conclusion that only magnesium (see Materials and Methods) might play a significant role in the branching of the two subpopulations (summit and toe). An important role of Ca^{2+} and Mg^{2+} has been shown for specific adsorption of *Rhizobium* species to legumes (29, 44). To date, no information is available about the possible effects of these ions on the rhizobial population structure. As described in Materials and Methods, a pH of 5.0 was measured in soil taken from slurry-polluted sites exposed to the north. Although pH-dependent changes in the rhizobial population structure have been described (4, 7, 12, 21, 46), we could not find any evidence for such effects.

When the PCR patterns of nodules derived from nonpolluted soil samples were analyzed separately (data not shown), slope position also exerted the major effect and exposure (north or south) had a smaller effect. In contrast, the effect of exposure (north or south) was not obvious after analyzing the slurry-polluted variants. These results suggest that the location

has a greater influence on the population structure in nonpolluted soil samples.

In conclusion, using a simplified ERIC-PCR technique applied directly to nodule extracts and followed by an automated computer analysis, we showed that special local factors and the exposure to bovine slurry affect the structure of the nodulating *R. leguminosarum* bv. viciae population in a natural environment, and we weighted the different factors with respect to their relative impact. The methods we used are excellent tools to characterize the nodulating *R. leguminosarum* bv. viciae population in the field, and the application of these techniques may be extended and used for a variety of bacterial populations for which appropriate markers are available.

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